Supporting Information
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Modified GSMP synthesis greatly improves the disulfide-crosslink of T7 run-off siRNAs with cell penetrating peptides

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Experimental Section:

E Chemicals were obtained from Fischer (chloroform p.a. stabilized with amylene), KFM Optichem (methanol p.a.), Sigma-Aldrich (trisodium thiophosphate, acetone p.a.), Riedel de Haen (formic acid) and Fluka (guanosine, MTPI, DMF-acetal, perchloric acid, water-free solvents). TLC-plates, Silicagel and LiChroprep RP-18 material (Merck, Darmstadt). Chromatography columns were purchased from Amersham Bioscience (DEAE Sepharose A-25) and from Knauer (Vertex Eurosphere-100). FPLC runs were carried out on instruments from Amersham Bioscience (Eatton LC and Äkta).

2’, 3’-O, O-Isopropylidene-5’-deoxy-5’-iodoguanosine, (2): Ground 2 (4.85 g, 15.0 mmol) is resuspended in dried THF (150 ml) under argon and cooled to −70°C (acetone and dry ice). Methyltriphenoxyphosphonium iodide (10.0 g, 22.1 mmol; 1.5 eq) is added. Due to the light sensitivity of the reactant and the product all subsequent steps have to be carried out under exclusion of light. After 30 min of stirring the reaction mixture is allowed to warm to room temperature and stirred for another 4 hours. The reaction is stopped by the addition of methanol (12 ml) and the solvent is removed under reduced pressure. The residue is resuspended in a mixture of diethyl ether and cyclohexane (1:1 (v/v)), filtered and thoroughly washed with diethylether and cyclohexane. The crude product is a bright yellow powder that is subsequently purified by column chromatography (silicagel: Merck 0.40-0.65 µm; eluent: CHCl3/MeOH 9:1 (v/v)). After removal of the solvent, 3 is obtained as a pale yellow powder in 38% yield (0.51 g, 1.18 mmol). To avoid side reactions that lead to a cyclization, moisture has to be strictly excluded in the first steps and the product should not be exposed to polar solvents. Tm: 134-136°C (degradation) - Rf = 0.80 (in CHCl3/MeOH 4:1 (v/v)); 0.32 (in CHCl3/MeOH 9:1 (v/v)). 1H NMR (300 MHz, CHCl3): δ [ppm] = 10.73 (s, 1H, NH), 7.89 (s, 1H, H-8), 6.56 (br, 2H, NH 2), 6.02 (d, J = 2.2 Hz, 1H, H-1´), 5.31 (dd, J = 3.1 Hz, 1H, H-3´), 5.03 (dd, J = 3.1 Hz, 1H, H-4´), 3.58 -3.46 (m, 2H, H-5´), 1.50 (s, 3H, CH 3), 1.30 (s, 3H, CH3).
Hz, 1H, H-5´), 3.33 (dd, J = 6.4 Hz, 10.0 Hz, 1H, H-5´), 1.50 (s, 3H, CH3), 1.31 (s, 3H, CH3). 13C NMR (101 MHz, d6-DMSO): δ [ppm] = 157.5, 154.6 (C-4, C-6), 151.3 (C-2), 137.2 (C-8), 114.2 (C-5), 113.2 (C-quart), 87.3, 84.7, 84.3, 81.1 (C-1’ to C-4´), 27.7 (CH3), 26.1 (CH3), 7.7 (C-5´). El (70 eV) m/z (%) = 433 (1%) [M+H]+, 305 (100%) [M-HI]+, 290 (13 %) [M-HI+CH3]+, 151 (20 %) [guanine]+, 128 (65%) [HI]+, 127 (31%), [I].

5´-Deoxy-5´-iodoguanosine (4): 3 (1.20 g, 2.78 mmol) (or an equivalent amount of 9) is resuspended in aqueous 50% formic acid (40 ml) and stirred for 3 days at room temperature under protection from light. The solvent is removed under reduced pressure at room temperature and the residue, 1.07 g (2.72 mmol, 98%) of a white solid, used for the next step without further purification. Tm = 142-145°C (degradation) - Rf = 0.26 (iPrOH/NH3/H2O, 6:3:1 (v/v/v)). - 1H NMR (400 MHz, D2O): δ [ppm] = 8.12 (s, 1H, H-8), 5.97 (d, J = 5.4 Hz, 1H, H-1´), 4.89 (dd, J = 5.4 Hz, 5.4 Hz, 1H, H-2´), 4.58 (dd, J = 4.1 Hz, 5.4 Hz, 1H, H-3´), 4.44 (dt, J = 4.1 Hz, 6.2 Hz, 1H, H-4´), 3.27 - 3.10 (m, 2H, H-5´). 31P NMR (162 MHz, D2O) = 19.0 ppm. - 13C NMR (101 MHz, D2O): δ [ppm] = 161.5, 156.4, 154.2 (C-2, C-4, C-6), 140.4 (C-8), 119.0 (C-5), 89.8, 87.2, 75.9, 74.7 (C-1´ to C-4´), 34.5 (C-5´). ESI-MS (negative mode, MeOH): 400 (2%) [M –2H + Na]+, 378 (100%) [M-H]–, HR-MS: 378.04 calculated: 378.027.

2´, 3´-O, O-Deoxysisopropylidine- 3, 5´-C-cycloguanosine (7): After 24 h at RT in d6-DMSO, 3 is completely converted into its cyclized form in an intramolecular reaction. 1H NMR (300 MHz, d6-DMSO): δ [ppm] = 8.62 (br, 1H, NH2), 8.07 (s, 1H, H-8), 6.52 (s, 1H, H-1´), 5.01 (d, J = 5.8 Hz, 1H, H-2´), 4.92 (t, J = 2.6 Hz, 1H, H-4´), 4.74 (dd, J = 2.6 Hz, 14.1 Hz, 1H, H-5´), 4.55 (d, J = 5.8 Hz, 8.5 Hz, 1H, H-3´), 3.98 (dd, J = 14.1 Hz, 2.6 Hz, 1H, H-5´), 1.45 (s, 3H, CH3), 1.24 (s, 3H, CH3). 13C NMR (101 MHz, d6-DMSO): δ [ppm] = 155.5, 153.6 (C-4, C-6), 140.6 (C-2), 136.9 (C-8), 112.8 (C-5), 113.2 (C-quart), 90.7, 85.5, 83.5, 80.7 (C-1´ to C-4´), 55.3 (C-5´), 26.1 (CH3), 24.7 (CH3). N,N-dimethylaminomethylene-2´,3´-O,O-isopropylidine-3,5´-C-cycloguanosine (10): After 10 days at RT in d6-DMSO, 9 has been completely converted into its cyclized form in an intramolecular reaction. 1H NMR (300 MHz, d6-DMSO): δ [ppm] = 8.39 (br, 1H, H-imine), 8.13 (s, 1H, H-8), 6.55 (s, 1H, H-1´), 5.30 (dd, J = 15.0 Hz, 2.7 Hz, 1H, H-5´), 4.90 (dd, J = 2.7 Hz, 2.7 Hz, 1H, H-4´), 4.88 (dd, J = 5.8 Hz, 1H, H-2´), 4.51 (d, J = 5.8 Hz, 1H, H-3´), 3.83 (dd, J = 15.0 Hz, 2.7 Hz; 1H, H-5´), 3.31 (s, 3H, N-CH3), 3.20 (s, 3H, N-CH3), 1.45 (s, 3H, C-CH3), 1.23 (s, 3H, C-CH3). 13C NMR (101 MHz, d6-DMSO): δ [ppm] = 159.8 (C-imine), 156.1, 155.3 (C-4, C-6), 141.1 (C-2), 137.3 (C-8), 121.9 (C-5), 112.8 (C-quart), 90.9, 85.7, 84.1, 80.7 (C-1´ to C-4´), 54.1 (C-5´), 42.1 (N-CH3), 36.2 (N-CH3), 26.2 (C-CH3), 24.8 (C-CH3).
Long-term NMR measurements

Long-term $^1$H NMR experiments are carried out on a Bruker spectrometer DPX500 at 500 MHz. The dried compounds are dissolved in d$_6$-DMSO immediately prior to measurement. Spectra are recorded in intervals of 30 min for 24 h and every 24 h for 20 days. The sets of spectra are phase corrected and integrated by the serial processing option of WinNMR 6.1 (Bruker). All integrals are calculated with reference to the DMSO signal. Further evaluation is carried out using Microsoft Excel 2000. The integrals of significant iodide signals, that showed no overlap with other signals over the course of the experiment, were normalized to the initial value and averaged. Correspondingly, the integrals of significant signals of the cyclized product were normalized to the end value. The averages were plotted against time with the recording of the first spectrum as t(0). The normalized integrals of the iodide (c$_\text{iodide}$) were plotted as their negative natural logarithm, - ln(c$_\text{iodide}$), against time and the rate constant k was obtained as the slope of the best fit straight line.

Mass spectroscopy

MALDI (Matrix assisted laser desorption ionization) spectra were obtained with a HiResMALDI FT-ICR (Ionspec, Lake Forrest, CA; 7 T magnet, pulsed laser 337 nm). pepsiRNAs were measured from a matrix of trihydroxyacetophenone (THAP) in the negative mode against a standard deoxyoligonucleotide of known mass. Samples were treated with ammonium acetate prior to treatment to exchange sodium and potassium ions for ammonium ions so that narrower peaks are obtained.

Reversed phase HPLC

The crude product is dissolved in bidistilled water and centrifuged (1 min, 14,000 rpm, 4°C, Centrifuge 5402, Eppendorf) to remove any precipitate. Up to 0.8 ml were loaded onto an RP-18 column (Nucleosil 120, Knauer) and purified by HPLC (Ettan, Amersham Bioscience). At flow rates of 0.8 ml/min the sample and side products are eluted with bidistilled degassed water. The fractions are immediately stored on ice. The run is monitored at 260 nm and the elution of the thiophosphate is detected the conductivity change.

siRNA preparation

21-nucleotide siRNA duplexes, containing a 5’ thiol modification, were either custom made (Dharmacon, USA) (GFP (synthetic) : 5’- HS-C$_6$-AAGCUGACCCUGAUUCAUC-3’) or enzymatically generated using T7 polymerase and the following DNA oligonucleotides as templates: T7 oligo: 5’S-TAAATACGACTCACTATAG-3’, GFP sense 1: 5’- AAGGTGGCATCGCCCTCAGTACCTATAG-3’, GFP antisense 5’- AAGGGCGAGGGCGATGCCCA-3’, GCS sense 1: 5’- AAGGACGGAGGGCGATGCCCA-3’, GCS antisense 1: 5’- AAGGACGGAGGGCGATGCCCA-3’. The underlined sequence encodes for the T7 binding site. The DNA oligonucleotides encoding either the sense or antisense strand (100 pmol each) were annealed with the T7 coding DNA oligonucleotide by boiling a 1:1 mixture and slowly cooling to room temperature (RT). The partially double stranded DNA was transcribed using the Ribomax T7-RNA polymerase system (Promega, USA) according to the manufacturers instructions with the following modifications: to generate the 5’- thiol modified sense RNA strand T7 reaction was carried out using an NTP mix that contained GSMP in an 8-fold excess of the other nucleotides. Following the reaction the strands are treated with DNase and alkaline phosphatase (CIP) (NEB) to remove the 5’-phosphate and to release the thiol function from the 5’-thiophosphate group. After 1 h of incubation at 37°C, the resulting sense and antisense 21 nt RNAs were precipitated for 1h at -20° (0.1 vol NaAc 3M, 2.5 vol ethanol). The RNA pellet is
dissolved in 10 mM Tris-HCl pH 8.5. Equimolar amounts of sense- and antisense-strand are pipetted together, heated to 70°C for 10 min and allowed to cool to RT over several hours for hybridization.

**Total RNA preparation and RT-PCR**
Cells were grown to confluency in 6x well plates. After removal of the medium cells were washed with 1x PBS and total RNA was prepared using the QIAGEN RNEasy RNA-extraction kit according to the manufacturer’s instructions. The RT-PCR was performed using the Titan One Tube RT-PCR system from Roche, Germany according to the manufacturer’s instructions. The following PCR conditions (PCR cycler MJ Research, USA) were used: 56°C for 35 min and 94°C for 2 min; 10 cycles of the following steps: 94°C for 20 s, 58°C for 45 s, 68°C for 2 min; 25 cycles of the following steps: 94°C for 20 s, 62°C for 45 s, 68°C for 2 min; end fill-up 68°C for 7 min. For RT-PCR analysis of residual mRNA in the RNAi cells the following primers were used: forward 5'-GGAGAATGGCGCTGCTGGACCTGGCCTTGGAG- 3' and reverse 5´-GGAGAAGAAACTGAAGAAATTGAATATGAGCCAG -3'; for pSAP control forward 5'- GGAGAATGTACGCCCTCTTCC TCCTGGCCAGC -3' and reverse 5'- GGAGAAGCGCACAGATCTCCTTGGGTTGCATG -3';

**SDS-PAGE**
Pentratin and pesiRNAs are separated and stained in non-reducing and reducing gels (15-20%) according to Laemmli et a[17]. Electrophoresis is performed in 6x8 cm vertical gels of 0.5-0.75 mm thickness in a Hoefer Minigel apparatus (Hoefer Mighty Small 250).

**Westernblot analysis.**
Wild type and pesiRNA treated were incubated at 37°C and 5% CO2 for 48 hours. After removal of the medium, the cells were washed with 1x PBS and lysed with 400 µl denaturing SDS-PAGE buffer. Homogenized samples were heated for 5 min at 95°C and separated in a 12.5% SDS-PAGE. Following SDS-PAGE, the gel was equilibrated for 20 min in transfer buffer (10 mM CAPS, pH 11 and 10% methanol) and blotted onto a PVDF membrane. Immunodetection of lamin A/C as a control was performed using a murine anti-lamin antibody (1:250-1000) (kindly provided by M. Osborn, Göttingen), as a primary antibody and an HRP-coupled anti rabbit-IgG (1:10.000). The respective proteins were detected by the HRP-activated chemiluminescence substrate using Lumiglo (Kirkkegaard)

**Generation of pesiRNAs**
A 20 nmol siRNA aliquot is dissolved in 120 µl of RNase free 0.1M DTT-solution (Titan-Kit, Roche) and incubated at 37°C overnight. The reduced siRNAs are purified from excess DTT and reaction products by gel filtration (MicroSpin™ G-25 Columns, AP-Biotech) according to the provider’s manual. Penetratin (Biotin-RQIKIWFQNRRMKWKKC-SH) (Qbiogene, France) was coupled according to the manufacturer’s instructions. A 13.5 nmol aliquot of the activated peptide is added to a 20 nmol (1.5 eq) aliquot of siRNA in 400 mM NaCl. The reaction mixture is incubated for 1 h at 37°C.

**Treatment of adherent cells with p esiRNA(synthetic) and p esiRNA(enzymatic) against GFP**
The pesiRNAs were dissolved in 10% DMEM to yield a 50 nM solution. 10⁴-10⁵ of the murine primary fibroblasts were plated into each well of a µ-slide 8-well ibiTreat (ibidi, Martinsried, Germany) and cultured in 200 µl of Dulbecco’s modified Eagle’s medium, high glucose, (DMEM, Sigma Taufkirchen) supplemented with 10% fetal calf serum (FCS, Gibco), and 1% Penicillin/Streptomycin at 37°C, 5% CO2. After the cells reach a confluency of about 80% the culture medium was removed and replaced by 0.2 ml of the respective pesiRNA supplemented media at a final concentration of 10-25nM. Knockdown of GFP was measured by live-cell imaging since fixation would alter the intracellular distribution of the Penetratin moiety as described for other polycationic species.

**Microscopy and Image Analysis.**
GFP fluorescence of the pesiRNA treated cells was measured after incubation for 48h, respectively by live imaging using a fluorescence microscope (Zeiss Axiovert 35, filter: Bp 546 FT 580, LP 590). For confocal microscopy cellular uptake was visualized using a confocal microscope (Zeiss Axiovert 200) fitted with a Zeiss LSM 510 Laser Module (argon, 458/488/514 nm; HeNe, 543/633 nm). Images were collected with the AxioCam MRc and the images were visualized with the AxioVision 3.1 and the LSM imaging software as well as the LSM Image Browser software version 3.1.0.99. Images were recorded with equal exposure times for specific.

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